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*Published in:*  
Environmental Microbiology

*Link to article, DOI:*  
[10.1111/1462-2920.12265](https://doi.org/10.1111/1462-2920.12265)

*Publication date:*  
2014

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*  
D'Alvise, P., Magdenoska, O., Melchiorson, J., Nielsen, K. F., & Gram, L. (2014). Biofilm formation and antibiotic production in *Ruegeria mobilis* are influenced by intracellular concentrations of cyclic dimeric guanosinmonophosphate. *Environmental Microbiology*, 16(5), 1252–1266. <https://doi.org/10.1111/1462-2920.12265>

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# Biofilm formation and antibiotic production in *Ruegeria mobilis* are influenced by intracellular concentrations of cyclic dimeric guanosinmonophosphate

Paul W. D'Alvise,\* Olivera Magdenoska,  
Jette Melchiorson, Kristian F. Nielsen and  
Lone Gram

Department of Systems Biology, Technical University of  
Denmark, Søltofts Plads, Bldg. 221, DK-2800 Kgs.  
Lyngby, Denmark.

## Summary

In many species of the marine *Roseobacter* clade, periods of attached life, in association with phytoplankton or particles, are interspersed with planktonic phases. The purpose of this study was to determine whether shifts between motile and sessile life in the globally abundant *Roseobacter* clade species *Ruegeria mobilis* are associated with intracellular concentrations of the signal compound cyclic dimeric guanosinmonophosphate (c-di-GMP), which in bacteria regulates transitions between motile and sessile life stages. Genes for diguanylate cyclases and phosphodiesterases, which are involved in c-di-GMP signalling, were found in the genome of *R. mobilis* strain F1926. Ion pair chromatography-tandem mass spectrometry revealed 20-fold higher c-di-GMP concentrations per cell in biofilm-containing cultures than in planktonic cells. An introduced diguanylate cyclase gene increased c-di-GMP and enhanced biofilm formation and production of the potent antibiotic tropodithietic acid (TDA). An introduced phosphodiesterase gene decreased c-di-GMP and reduced biofilm formation and TDA production. *tdaC*, a key gene for TDA biosynthesis, was expressed only in attached or biofilm-forming cells, and expression was induced immediately after initial attachment. In conclusion, c-di-GMP signalling controls biofilm formation and biofilm-associated traits in *R. mobilis* and, as suggested by presence of GGDEF and EAL domain protein genes, also in other *Roseobacter* clade species.

## Introduction

The *Roseobacter* clade (*Alphaproteobacteria*) accounts for a significant part of the microbiota in the oceans, especially in coastal zones and surface waters (Gonzalez and Moran, 1997; Buchan *et al.*, 2005; Brinkhoff *et al.*, 2008; Newton *et al.*, 2010; Wietz *et al.*, 2010). *Roseobacter* clade species are metabolically and ecologically diverse, comprising aerobic anoxygenic phototrophs, sulphur metabolizers, carbon monoxide oxidizers and degraders of aromatic compounds (Shiba *et al.*, 1979; Sorokin and Lysenko, 1993; Moran and Hodson, 1994; Buchan *et al.*, 2001; Allgaier *et al.*, 2003; Moran *et al.*, 2003; 2004). However, most species of the *Roseobacter* clade are classified as ecological generalists (Moran *et al.*, 2004; Newton *et al.*, 2010). Abundance and activity of many *Roseobacter* clade members are correlated with phytoplankton population densities, and one prominent ability of many *Roseobacter* clade members is the conversion of the phytoplankton osmolyte dimethylsulfoniopropionate to the volatile dimethyl sulphide, which influences the local and global climate (Charlson *et al.*, 1987; Gonzalez and Moran, 1997; Gonzalez *et al.*, 2000; Moran *et al.*, 2003; Geng and Belas, 2010b).

Belas and colleagues (2009) noticed that many *Roseobacter* clade species have a 'biphasic swim-or-stick lifestyle' that enables their symbiosis with phytoplankton, and suggested that a central regulation mechanism coordinated the shift between planktonic and attached phenotype. Accumulating evidence indicates that bis-(3'-5')-cyclic dimeric guanosinmonophosphate (c-di-GMP) functions as a nearly universal second messenger in bacteria, regulating transitions between planktonic and sedentary phases by controlling phenotypic features such as flagellar motility and production of extracellular polymeric substances (EPS) (Hengge, 2009; McDougald *et al.*, 2012). Above that, c-di-GMP regulates important functions that are associated with either one of the lifestyles, such as virulence or antibiotic production (Schmidt *et al.*, 2005; Cotter and Stibitz, 2007; Tamayo *et al.*, 2007). The intracellular pool of c-di-GMP is balanced by diguanylate cyclases (GGDEF-domain proteins) that synthesize the compound and by specific phosphodiesterases (EAL-domain proteins) that degrade it (Ausmees *et al.*, 2001;

Received 10 May, 2013; revised 19 August, 2013; accepted 26 August, 2013. \*For correspondence. E-mail pdal@bio.dtu.dk; Tel. (+45) 45255501; Fax (+45) 45884922.

Ryjenkov *et al.*, 2005; Gjermansen *et al.*, 2006). The activity of these antagonistic enzymes is controlled by sensory domains or proteins that allow external or internal stimuli to act on the intracellular pool of c-di-GMP and thus influence the decision between sessile and motile life. Here, we hypothesized that the transition between planktonic and attached lifestyle in *Roseobacter* clade species is induced by intracellular c-di-GMP levels. To test this, we introduced the plasmids pYedQ and pYhjH that have been used as tools to demonstrate that c-di-GMP signalling regulated *Pseudomonas putida* biofilm formation and dispersal (Gjermansen *et al.*, 2006). The plasmids contain either one of the *Escherichia coli* genes *yedQ* and *yhjH*, which encode a diguanylate cyclase that synthesizes c-di-GMP and a c-di-GMP-degrading phosphodiesterase respectively. Intracellular levels of c-di-GMP in *Ruegeria mobilis* F1926 wild-type and plasmid-carrying mutants were assessed using ion pair liquid chromatography-tandem mass spectrometry (Ion-Pair UHPLC-MS/MS).

The *Roseobacter* clade genera *Ruegeria* and *Phaeobacter* have been of particular interest due to their ability to form the potent antibacterial compound tropodithietic acid (TDA). *Phaeobacter* strains have been isolated from coastal zones, especially from biofilms in fish and invertebrate larvae cultures, and have been studied for their antibacterial activity and as probiotics for marine aquaculture (Ruiz-Ponte *et al.*, 1998; Brinkhoff *et al.*, 2004; Hjelm *et al.*, 2004; Planas *et al.*, 2006; Porsby *et al.*, 2008; D'Alvise *et al.*, 2010). *Ruegeria* (*Silicibacter*) sp. TM1040 is a symbiont of the dinoflagellate *Pfisteria piscicida* and was the first TDA-producing bacterium in which parts of the TDA biosynthetic pathway were elucidated (Alavi *et al.*, 2001; Miller and Belas, 2006; Bruhn *et al.*, 2007; Moran *et al.*, 2007; Yi *et al.*, 2007; Geng *et al.*, 2008; Geng and Belas, 2010a). *R. mobilis* (*Ruegeria pelagia*) has been isolated in surface ocean waters in most climatic zones, except Arctic and Antarctic waters, and it occurs in both coastal zones and open oceans (Gram *et al.*, 2010; Lai *et al.*, 2010). Several strains of *R. mobilis*, which all produce TDA, have been isolated from microalgae cultures, highlighting their preference for phytoplankton (Porsby *et al.*, 2008).

TDA production is influenced by culture conditions, and Bruhn and colleagues (2005) found that TDA production by *Phaeobacter* sp. 27-4 in Marine Broth (MB; Difco Laboratories, Detroit, MI, USA) only occurred under static growth conditions but not in shaken broth cultures. TDA production coincided with formation of a thick layer of biofilm at the air-liquid interface consisting of multicellular, star-shaped aggregates. To our knowledge, *Ruegeria* strains can only produce TDA in static liquid cultures, where a biofilm at the air-liquid interface is formed, whereas most *Phaeobacter* strains are able to produce TDA in shaken liquid cultures as well. This makes

*Phaeobacter* sp. 27-4 an exemption among the TDA-producing *Phaeobacter* strains (Porsby *et al.*, 2008). A later study, also using strain 27-4, demonstrated that attachment to an inert surface was affected by culture conditions as well (Bruhn *et al.*, 2006). However, although the tight association between biofilm formation and TDA production strongly indicates that TDA is only produced by attached or biofilm-forming cells, this was never verified. In the present study, we demonstrate that c-di-GMP plays a major role in the transition from motile to sessile state in *R. mobilis*, and this led us to hypothesize that formation of TDA, which is associated with biofilms, could be correlated with c-di-GMP. Consequently, investigating whether TDA production was affected by altered c-di-GMP levels became a second aim of this study. Therefore, we measured the impact of changed c-di-GMP levels on antibacterial activity and TDA production and studied the expression of the *tdaC* gene in attached and planktonic cells.

## Results

### Bioinformatic analysis

The genome of *R. mobilis* F1926 was Illumina-sequenced and assembled into 1065 contigs with a total length of about 4.5 Mb. One hundred ten contigs were larger than 10 kb and contained together 2.5 Mb. For comparison, the closest genome-sequenced relative (on 16S-rRNA-gene-level) of strain F1926, *Ruegeria* sp. TM1040 (Supporting Information Fig. S1), contains 4.2 Mb of genomic DNA, a megaplasmid of 0.8 Mb and a large plasmid of 0.1 Mb (Moran *et al.*, 2007). Nine genes encoding diguanylate cyclases and c-di-GMP-specific phosphodiesterases were identified in *R. mobilis* F1926 based on Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) annotation, which was manually controlled by Pfam classification (Table 1). Six of these encoded proteins that contained both a GGDEF and an EAL domain. This could indicate that these enzymes have alternating c-di-GMP synthesizing or degrading activity; however, there are examples of proteins that contain both GGDEF and EAL domains, but act only as either diguanylate cyclase or phosphodiesterase, or have no c-di-GMP converting activity at all, but act as signalling proteins (Christen *et al.*, 2005; Matilla *et al.*, 2011; Newell *et al.*, 2011).

### c-di-GMP analysis by ion pair UHPLC-MS/MS

The plasmids pYedQ and pYhjH, as well as the respective vector controls pRK404A and pBBR1MCS-3, were introduced into *R. mobilis* F1926 with the aim of manipulating intracellular c-di-GMP concentrations, which was

**Table 1.** GGDEF- and EAL-domain protein genes identified in *Ruegeria mobilis* F1926.

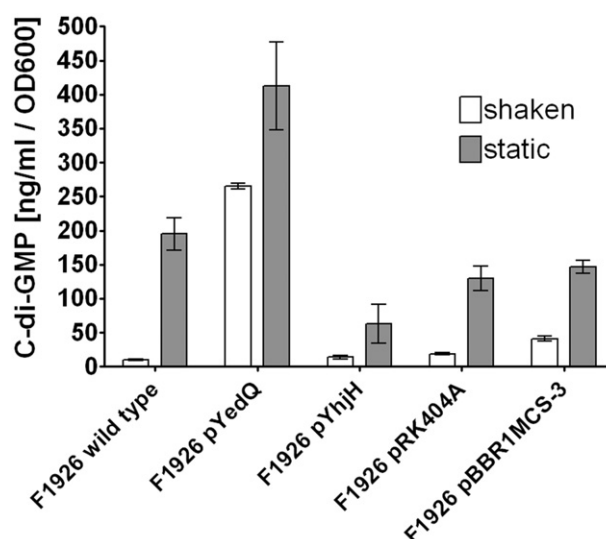
Locus tag	Predicted gene product	Pfam domain	E-value (Pfam)	Length [aa]
K529_01085	diguanylate cyclase	GGDEF	$4.1 \times 10^{-29}$	313
K529_06935	diguanylate cyclase/phosphodiesterase	GGDEF	$2.5 \times 10^{-35}$	684
		EAL	$5.4 \times 10^{-66}$	
K529_19462	diguanylate cyclase/phosphodiesterase	GGDEF	$1.7 \times 10^{-45}$	700
		EAL	$1.7 \times 10^{-59}$	
K529_09228	diguanylate cyclase	GGDEF	$3.3 \times 10^{-39}$	342
K529_12495	diguanylate cyclase domain-containing protein	GGDEF	$2.8 \times 10^{-34}$	674
		EAL	$6.4 \times 10^{-67}$	
K529_13194	diguanylate cyclase/phosphodiesterase	GGDEF	$6.9 \times 10^{-6}$	393
		EAL	$7.6 \times 10^{-57}$	
K529_15333	diguanylate cyclase/phosphodiesterase	GGDEF	$7.7 \times 10^{-23}$	496
		EAL	$2.3 \times 10^{-57}$	
K529_15543	diguanylate cyclase/phosphodiesterase ammonium transporter	GGDEF	$5.7 \times 10^{-35}$	904
		EAL	$4.4 \times 10^{-59}$	
		Ammonium_transp	$1.8 \times 10^{-96}$	
K529_20992	response regulator receiver modulated diguanylate cyclase	GGDEF	$8.0 \times 10^{-46}$	467

subsequently verified by ion pair chromatography-MS/MS in extracts of 24 h old shaken and static MB cultures. The compound was detected in all extracts with the same retention time as an authentic standard. A chromatographic plot of a standard and a sample is given in the Supporting Information Fig. S2. Concentrations in the extracts ranged from 26 to 770 nM and were divided by the optical density (OD<sub>600</sub>) of the original culture to obtain a relative measure of c-di-GMP per cell (Fig. 1). Care was taken to break up the biofilms of the static cultures before sampling and OD measurement to allow comparison of cell density to shaken cultures. Static cultures of the wild type, where thick air liquid interface biofilms were observed, contained 20 times more c-di-GMP per cell than shaken cultures, where no biofilms were formed. Plasmid pYedQ increased c-di-GMP per cell to the 30-fold concentration of the vector control under shaken conditions ( $P < 0.001$ ) and to the threefold concentration in static cultures ( $P < 0.001$ ). In the static cultures of the mutant carrying plasmid pYhjH, c-di-GMP concentration per cell was reduced to about half of the respective vector control; however, this was not significant ( $P > 0.05$ ), and under shaken conditions, no further reduction in c-di-GMP was found. Regardless of the introduced plasmids, cultivation conditions influenced c-di-GMP levels. For all strains, c-di-GMP concentrations per cell were higher in static cultures than in shaken cultures ( $P < 0.001$ ).

#### Phenotypic effects of altered c-di-GMP levels

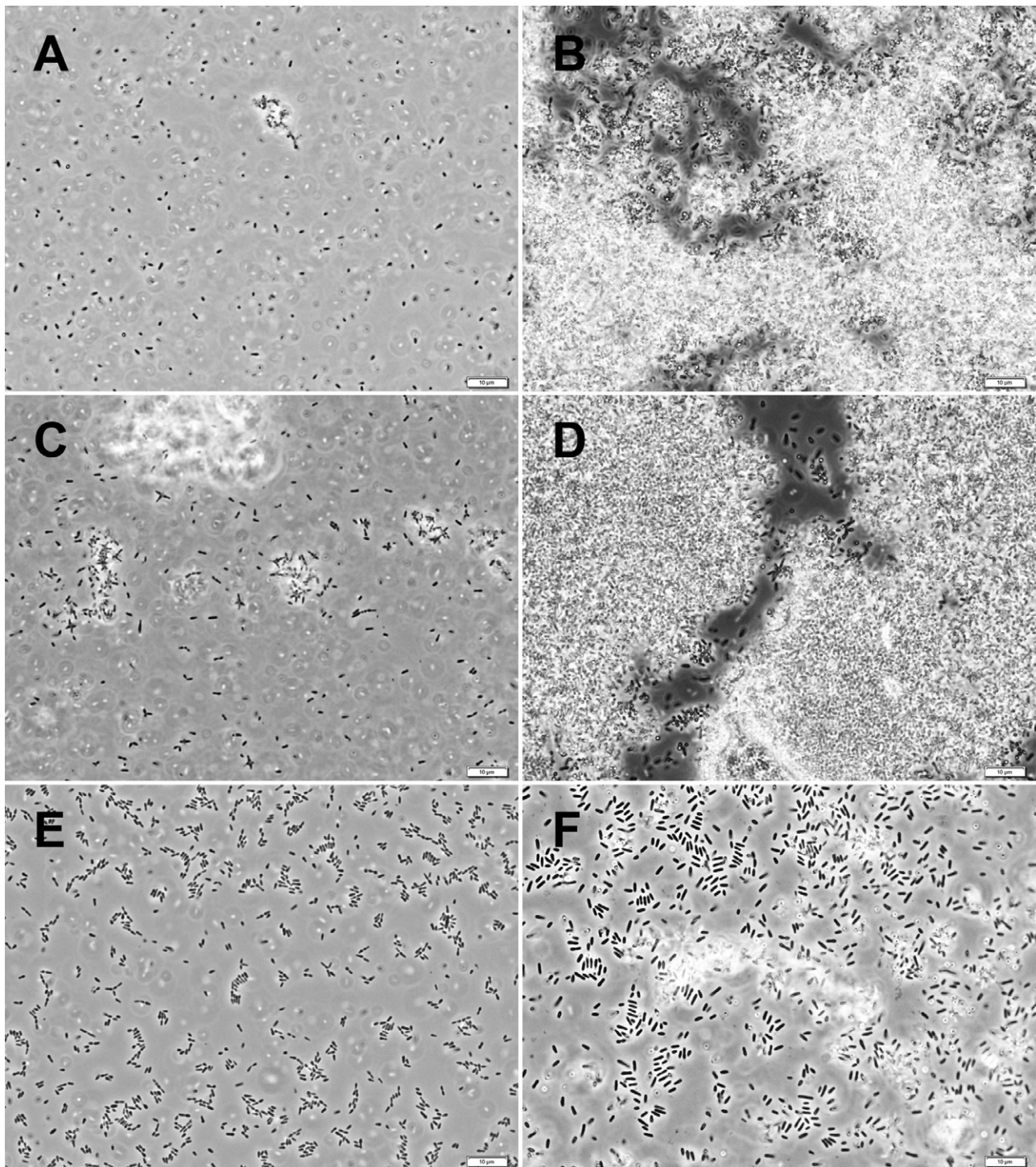
Shaken cultures of *R. mobilis* F1926 wild type were dominated by single cells, and about half of these were motile (Fig. 2A). Introduction of pYedQ, which increased c-di-GMP concentrations, caused disappearance of motile cells and increased formation of multicellular aggregates in shaken cultures (Fig. 2C). *R. mobilis* F1926 pYhjH,

which contained less c-di-GMP, grew almost exclusively as single motile cells in shaken cultures (Fig. 2E). Static cultures of the wild type were dominated by biofilms consisting of multicellular, star-shaped aggregates (rosettes), but also, motile single cells were present (Fig. 2B). In static cultures of the pYedQ-carrying mutant, even thicker rosette-containing biofilms were formed, and no motile cells were observed (Fig. 2D). Introduction of pYhjH increased the proportion of motile cells and prevented formation of rosettes in static cultures (Fig. 2F). However, no difference in motility on population level could be detected in a motility-agar test (data not shown).



**Fig. 1.** Cyclic di-GMP concentrations in extracts of 24 h old shaken and static cultures of *Ruegeria mobilis* F1926 wild type, F1926 pYedQ, F1926 pYhjH and the respective vector control strains F1926 pRK404A and F1926 pBBR1MCS3, divided by OD<sub>600</sub> of the cultures. Given values are averages of three independent replicates.

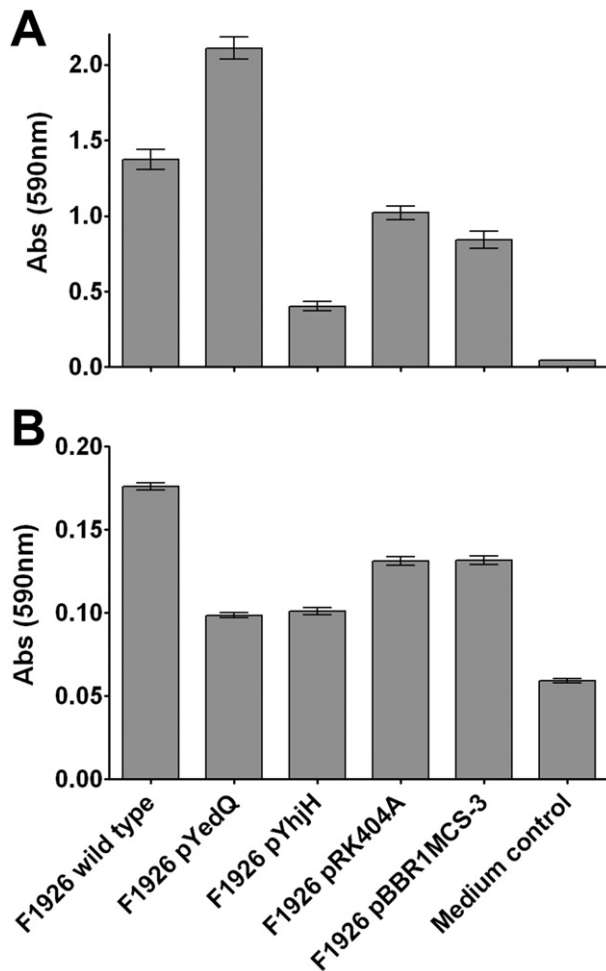




**Fig. 2.** Phase-contrast micrographs of shaken (A, C, E) and static (B, D, F) Marine Broth cultures of *Ruegeria mobilis* F1926 wild type (A, B), F1926 pYedQ (C, D) and F1926 pYhjH (E, F). All scale bars indicate 10 µm. The vector control strains were similar to the wild type (not shown).

Biofilm formation and attachment were assessed in the plasmid-carrying strains and wild type (Fig. 3). Biofilm formation (Fig. 3A) was significantly increased in F1926 pYedQ ( $P < 0.001$ ), whereas in F1926 pYhjH, it was

reduced as compared with the respective vector controls and the wild type ( $P > 0.001$ ). Attachment of planktonic cells from shaken cultures was measured as stained biomass of cells attaching to polystyrene pegs within



**Fig. 3.** Biofilm formation (A) and attachment (B) of *Ruegeria mobilis* F1926 wild type, F1926 pYedQ and F1926 pYhjH, and the respective vector control strains F1926 pRK404A and F1926 pBBR1MCS3, measured in crystal violet assays. Cells from shaken cultures were used in the attachment assay. Sterile medium (1.5 YTSS) was used as control. Given values are averages of two independent replicates.

1 min (Fig. 3B). The wild type attached most efficiently, whereas the attachment was reduced in both the pYhjH-carrying strain and the strain carrying pYedQ, indicating that both a decrease and an increase of intracellular c-di-GMP levels interfere with attachment. Although attachment was also reduced in the vector controls, which might be an effect of the tetracycline in the medium, the reduction of attachment conferred by the introduced GGDEF- and EAL-domain proteins was significant ( $P < 0.001$ ).

The antibacterial effect of the plasmid-carrying mutants and the wild type was compared in agar-diffusion-inhibition assays, and TDA production was measured by ultra-performance liquid chromatography high-resolution mass spectrometry (UHPLC-TOFMS). In static cultures, the pYedQ-carrying mutant caused inhibition zones of equal or larger size than the wild type and vector controls,

whereas the mutant carrying pYhjH was less inhibitory (Table 2). As opposed to the wild type, vector controls and the pYhjH-carrying mutant, the pYedQ-carrying mutant caused inhibition also in shaken cultures. As indicated by the inhibition test, TDA production was influenced by intracellular c-di-GMP concentrations (Fig. 4). Brown pigmentation of the cultures, which is indicative of TDA production (Bruhn *et al.*, 2005), was correlated with inhibition and TDA production (not shown).

#### Expression of *tdaC* on single-cell level

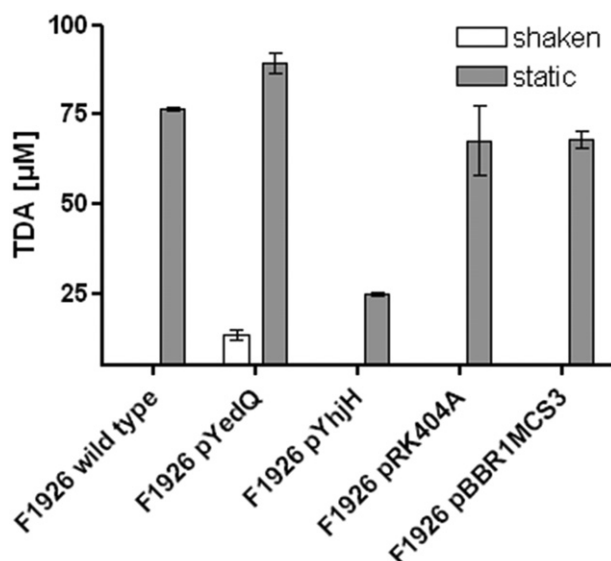
TDA production has been suggested to be regulated on community level by auto-induction (Geng and Belas, 2010a) or by quorum sensing (QS) (Berger *et al.*, 2011). However, the observation that inhibitory activity was influenced by intracellular c-di-GMP levels, which can be different between attached and planktonic cells of the same community, suggested that TDA production could differ in cells within the same culture. This prompted us to study the expression of a key gene involved in TDA biosynthesis (*tdaC*) on single-cell level by using a promoter-*gfp* fusion.

In shaken cultures, *R. mobilis* F1926 pPDA11 (*PtdaC::gfp*) cells were predominantly planktonic (Fig. 5B), and approximately half the cells were motile, as described above for the wild type. *tdaC* was not expressed, as indicated by lack of green fluorescence (Fig. 5A and B), correlating with a lack of antibacterial activity and TDA formation (Table 2, Fig 4). Yet a few small multicellular aggregates in which *tdaC* was expressed were found (Fig. 5A and B). In contrast, major proportions of the cells from static cultures were situated in biofilms or multicellular aggregates and expressed the *tdaC* gene (Fig. 5C and D). However, a part of the single cells in the samples from static cultures was not fluorescent, and a fraction of these was motile. No expression of *tdaC*, as indicated by green fluorescence, was observed in motile cells. This indicated that *tdaC*, a gene encoding a central enzyme in the TDA biosynthesis pathway, is expressed differently in adjacent cells within the same cultures, and

**Table 2.** Inhibition of *V. anguillarum* 90-11-287 by cell-free supernatants of *R. mobilis* F1926 wild type, F1926 pYedQ, F1926 pYhjH, F1926 pRK404A and F1926 pBBR1MCS-3 cultures, grown in shaken (200 r.p.m.) or static MB for 72 h at 25°C.

Strain	Inhibition zone diameter without well diameter [mm] ± standard deviation	
	Shaken cultures	Static cultures
<i>R. mobilis</i> F1926 wild type	0 ± 0	13.5 ± 0.7
<i>R. mobilis</i> F1926 pYedQ	1.5 ± 0.7	15 ± 0
<i>R. mobilis</i> F1926 pYhjH	0 ± 0	8 ± 1.4
<i>R. mobilis</i> F1926 pRK404A	0 ± 0	12 ± 0
<i>R. mobilis</i> F1926 pBBR1MCS-3	0 ± 0	12 ± 1.4





**Fig. 4.** Concentrations of TDA in shaken and static cultures of *Ruegeria mobilis* F1926 wild type, F1926 pYedQ, F1926 pYhjH and the respective vector control strains F1926 pRK404A and F1926 pBBR1MCS3, grown in shaken or static MB for 72 h at 25°C. Values are averages of two independent replicates.

that its expression coincides with attachment and is enhanced in biofilms or aggregates where high c-di-GMP levels are found.

#### Induction of *tdaC* expression in response to attachment

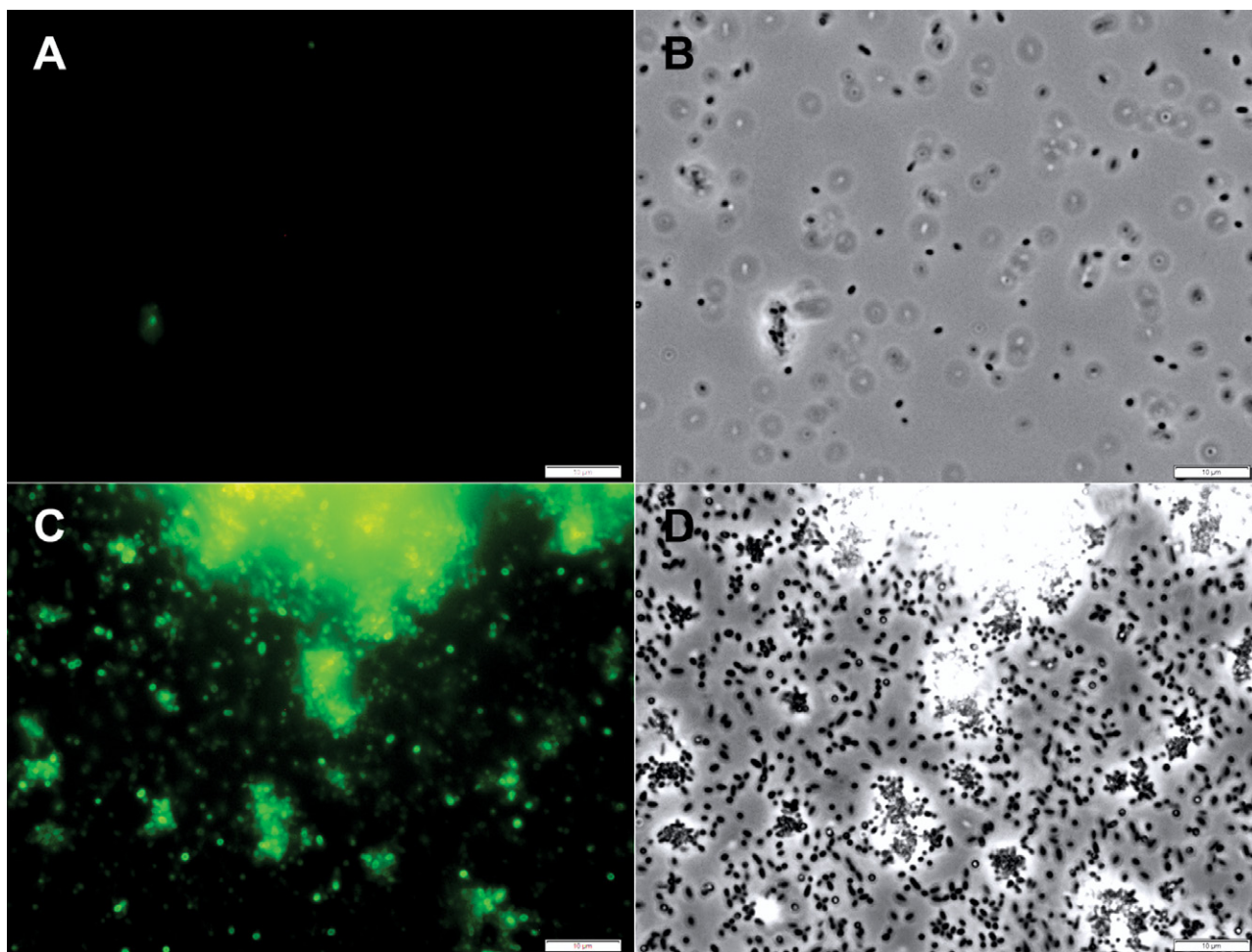
When a sample of a shaken *R. mobilis* F1926 pPDA11 culture was prepared for microscopy, some of the non-motile single cells attached immediately to the cover glass. A time series of fluorescence and phase contrast micrographs showing newly attached cells on the cover glass was recorded (Fig. 6). *Gfp* expression was initiated in the newly attached cells, and green fluorescence could be observed already 10 min after preparation of the microscope slide. Maximal fluorescence of the initially attached cells was reached about 15–20 min after start of the experiment. Considering the time needed for *gfp* gene expression and maturation, this suggests that expression was initiated immediately when the cells attached. This instant initiation of *tdaC* expression may indicate a regulatory connection between TDA production and initial attachment, which is likely accompanied by rising c-di-GMP levels.

#### Discussion

Attachment to surfaces and biofilm formation are characteristic features of many *Roseobacter* clade species, and a more comprehensive understanding of the transition between motile and sessile life stages in the *Roseobacter*

clade is needed to understand carbon and nutrient cycling in the oceans (Slightom and Buchan, 2009). Also, production of the antibacterial substance TDA in *Phaeobacter* spp. has been associated with biofilms (Bruhn *et al.*, 2005), and since TDA is a key component for their probiotic effect on fish larvae (D'Alvise *et al.*, 2012), understanding the transition between motile and sessile stages may provide new perspectives on their application.

Many bacteria have two distinct lifestyles, a sessile or biofilm stage on a substrate or within a host, which is mostly characterized by increased metabolic activity and proliferation, and a mobile stage, where the cells are metabolically less active, disperse into the wider environment and persist until a new substrate or host is found. The transition between these two distinct states is in many bacteria controlled by a pool of intracellular c-di-GMP that exerts control on every level of regulation. C-di-GMP binds to transcriptional regulators and also exerts control at translational and allosteric levels, regulating a multitude of phenotypic traits, e.g. motility, EPS production, extracellular appendage formation, virulence or production of secondary metabolites (Hengge, 2009; McDougald *et al.*, 2012). The results of our study suggest that c-di-GMP signalling plays a similar role in *R. mobilis*. Intracellular concentrations of c-di-GMP changed with cultivation conditions that favoured or prevented biofilm formation. Manipulation of c-di-GMP levels seemed to alter the proportions of motile and sessile cells under different culture conditions and affected expression of phenotypes that are associated with either planktonic or attached lifestyle. Increased levels of c-di-GMP promoted biofilm formation, whereas a decrease in c-di-GMP concentrations prevented formation of star-shaped aggregates and reduced TDA production. The finding that an introduced GGDEF domain protein increased biofilm formation and interfered with motility is consistent with previous studies (Ausmees *et al.*, 2001; Simm *et al.*, 2004; Gjermansen *et al.*, 2006; Wolfe and Visick, 2008; Hengge, 2009; McDougald *et al.*, 2012) and indicates a functional c-di-GMP signalling system in *R. mobilis*. This is confirmed by presence of genes encoding GGDEF and EAL domain proteins in the genome of *R. mobilis* F1926. Presence of similar genes annotated as diguanylyl cyclases and c-di-GMP-specific phosphodiesterases in the closely related strains *Ruegeria* sp. TM1040, *Ruegeria* sp. R11 and *Phaeobacter gallaeciensis* DSM17395, as well as in more remote *Roseobacter* clade species such as *Roseobacter litoralis* Och149, *Roseovarius* sp. 217, *Octadecabacter arcticus* 238 and *Dinoroseobacter shibae* DFL12 (Supporting Information Table S1), suggests that c-di-GMP signalling is a universal feature of the *Roseobacter* clade. We conclude that in the *Roseobacter* clade, in analogy with many other bacteria, intra and



**Fig. 5.** Expression of *tdaC* in *Ruegeria mobilis* F1926 grown in shaken (A, B) and static (C, D) MB cultures, monitored using a plasmid-bound reporter fusion of the *tdaC*-promoter with a promoterless *gfp*-gene (pPDA11). Fluorescence (A, C) and phase contrast (B, D) micrographs were each recorded using the same settings.

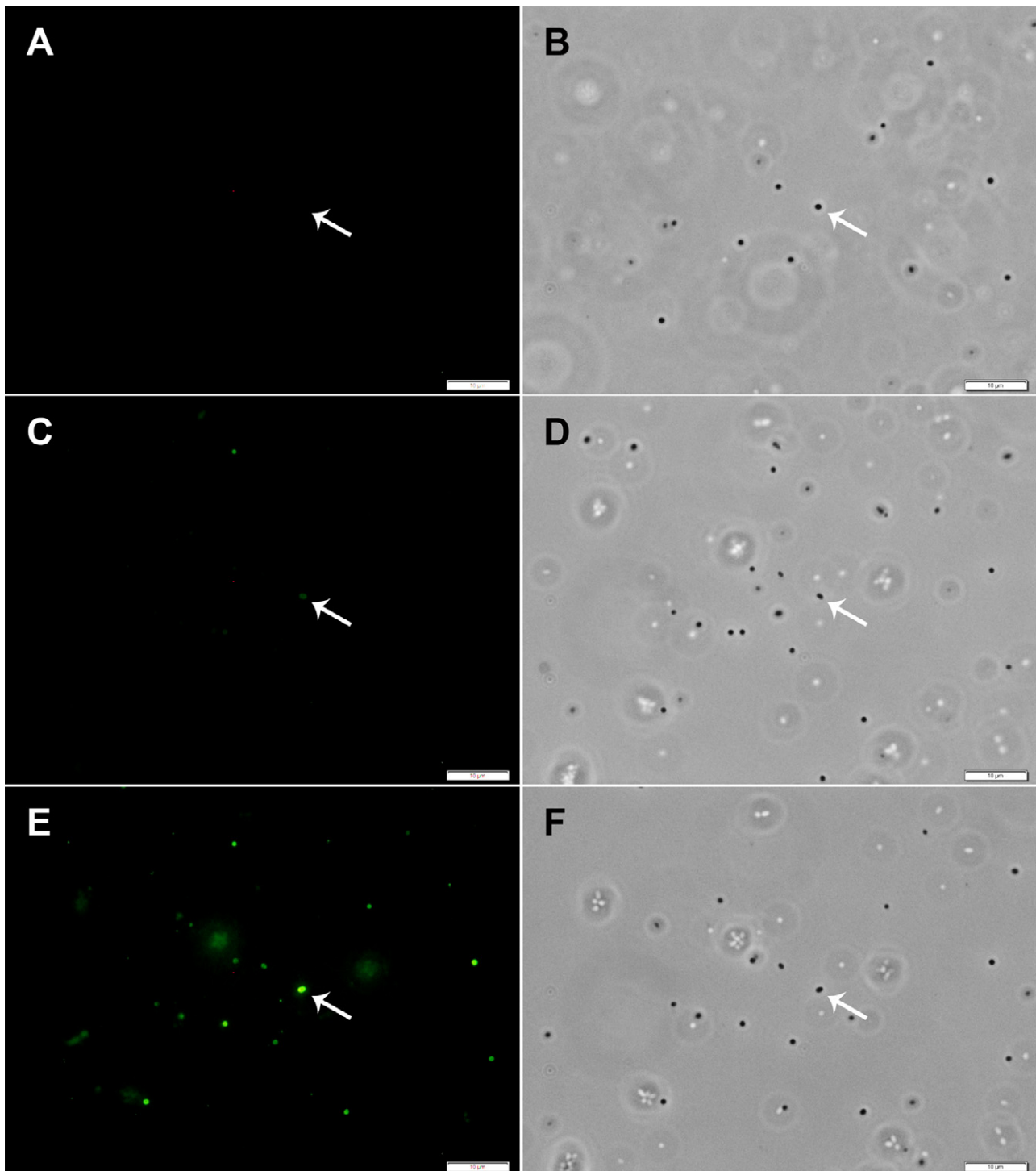
extracellular cues are integrated via a c-di-GMP second messenger system and that expression of phenotypic traits specific for either planktonic or attached life is regulated in response to c-di-GMP concentrations. Belas and colleagues (2009) have introduced the term 'swim-or-stick switch' for the molecular mechanism that regulates transitions between motile and sessile stage in *Roseobacter* clade species. We think that the intracellular concentration of c-di-GMP is the swim-or-stick switch.

Recently, Zan and colleagues (2012) revealed that in *Ruegeria* sp. KLH11, a sponge symbiont, motility and biofilm formation are controlled by N-acyl homoserine lactone-based QS. Similarly, Sule and Belas (2012) found that in *Ruegeria* sp. TM1040 motility and biofilm formation are controlled by a QS-like system based on a diffusible signal compound with a molecular mass of about 226 Da. *R. mobilis* strain F1926 may utilize the same or a similar QS system as *Ruegeria* sp. TM1040, and since in this case QS and c-di-GMP signalling control the same phe-

notypes, the two regulation systems are likely connected at some level. In a review of connections between QS and c-di-GMP signalling, Srivastava and Waters (2012) propose that QS signals are generally integrated into the epistatic c-di-GMP signalling system, allowing information about local cell density to be merged with other environmental cues for making a decision between attached and planktonic life. Thus, studying the connection between QS and c-di-GMP signalling may provide further insight into how motility and biofilm formation are controlled.

Most studies of c-di-GMP signalling have approached the role of the compound using bioinformatic and transcriptional tools or genetic manipulation. Few studies actually measured concentrations of c-di-GMP to substantiate their findings (e.g., Weinhouse *et al.*, 1997; Waters *et al.*, 2008; Merritt *et al.*, 2010; Spangler *et al.*, 2010). However, due to the ionic nature of c-di-GMP, it used to be difficult to obtain reproducible retention times, as well as sharp symmetrical peaks using conventional





**Fig. 6.** Time series of *tdaC* expression in newly attached *Ruegeria mobilis* F1926 pPDA11. The images were recorded 1 min (A, B), 10 min (B, C) and 20 min (E, F) after preparing the specimen. Fluorescence (A, C, E) and phase contrast (B, D, F) micrographs of the same area were each recorded using the same settings. The focal plane was set right beneath the cover slip to record attached cells. White arrows indicate the position of the same attached cell in all images. Right after preparing the specimen, the freshly attached cells were not fluorescent (A), yet green fluorescence indicating expression of *tdaC* was observed in attaching cells after 10 min. All scale bars indicate 10  $\mu$ m.

reversed phase chromatography (Werner, 1991; Simm *et al.*, 2004). LC-MS/MS with tributylamine as ion pair reagent was chosen due to its sensitivity and higher volatility of the tributylamine (Magdenoska *et al.*, 2013) when compared with tetrabutylammonium (Witters *et al.*, 1997) which reduces background and ion-source pollution (Holcapek *et al.*, 2004).

A relative of the *Roseobacter* clade within the  $\alpha$ -Proteobacteria, *Caulobacter crescentus*, has a very sophisticated variant of c-di-GMP-mediated 'swim-and-stick' life (Viollier *et al.*, 2002; Aldridge *et al.*, 2003; Paul *et al.*, 2004; Huitema *et al.*, 2006; Duerig *et al.*, 2009; Abel *et al.*, 2011). The cell cycle comprises a stage of flagellated swarmer cells, in which replication is inhibited via low c-di-GMP levels, and a stage of sessile, stalked cells that form new swarmer cells at their non-attached end. In the stalked cells, c-di-GMP is unequally distributed as an effect of antipodal location of GGDEF and EAL domain proteins, restricting cell division to the non-attached pole. In the present study, star-shaped rosettes were not formed in a mutant with lowered c-di-GMP levels, indicating an involvement of the compound in producing that phenotype. Rosette formation in *Roseobacter* clade species could be the result of a process involving polar differences in c-di-GMP contents, where cell division is possibly restricted to one pole of the rosette-forming cell and its daughter cells.

Bruhn and colleagues (2007) demonstrated that cells of *Phaeobacter* sp. 27-4 from static cultures attached better to a glass surface than cells from shaken cultures. The same pattern was observed in this study. However, comparing attachment between shaken cultures of wild type and mutants with altered c-di-GMP levels, we found that initial attachment was compromised both by increased and decreased c-di-GMP levels. Miller and Belas (2006) demonstrated that in *Ruegeria* sp. TM1040, motility is crucial for initiating the *Ruegeria*-dinoflagellate symbiosis. Consequently, the strain with increased c-di-GMP levels may have a reduced capability of attaching to substrates because motility was repressed. Interestingly, attachment of the pYhJH-carrying strain with decreased c-di-GMP and increased motility was reduced as well, even if not to the same extent. This might be caused by a reduced ability to turn off flagellar motility in response to initial surface contact by c-di-GMP-mediated allosteric inhibition of the flagellar motor as known from *C. crescentus* (Christen *et al.*, 2007).

Antibacterial activity, production of the brown pigment and TDA production were reduced by decreased intracellular c-di-GMP levels, suggesting that the association between the biofilm phenotype and TDA production could be c-di-GMP-mediated. This led us to study expression of *tdaC* as indicator of TDA production on single-cell level, and we found that *tdaC* is expressed differently within

different cells of the same cultures. This argues against a regulation mechanism based exclusively on community level. Two regulation mechanisms for TDA production were identified on community level. QS was found to activate production of TDA in *P. gallaeciensis* (Berger *et al.*, 2011), and also TDA itself was observed to act as an autoinducer, causing increased expression of genes necessary for its own production (Geng and Belas, 2010a). However, both mechanisms fail to explain how *tdaC* can be expressed differently in adjacent cells. The hypothesis that TDA production is regulated by c-di-GMP provides an alternative explanation for how TDA production can be different in cells within the same culture, and for how it can, on single-cell level, be spontaneously induced by attachment despite absence of TDA.

The *tdaC* gene is expressed only in biofilms or aggregates, and its expression could be initiated in planktonic cells by physical attachment to a surface. Thus, *tdaC* was expressed where high or rising levels of c-di-GMP would probably be found. Geng and Belas (2011) showed that the TdaA protein, a LysR-type transcriptional regulator, binds to the *tdaC* promoter and activates *tdaC* expression. The ligands of LysR-type transcriptional regulator are small molecules (Schell, 1993), and it could be speculated whether c-di-GMP is the ligand of TdaA, or whether intermediate steps are involved.

In conclusion, our study adds organisms from the *Roseobacter* clade to the list of bacteria that use c-di-GMP as a key secondary messenger. Notably, c-di-GMP may be the key molecule in the often described 'stick-and-swim' lifestyle of several roseobacters.

## Experimental procedures

### *Strains, plasmids and media*

An overview of strains and plasmids is provided in Table 3. *R. mobilis* F1926 was isolated from the central Indian Ocean (coordinates -31.4061, 91.17758) during the Galathea III expedition and was identified by its 16S rRNA gene sequence (L. Gram, P. D'Alvise, C. Porsby, J. Melchiorson, J. Heilmann, M. Jensen, *et al.*, unpublished data) using procedures described in Gram and colleagues (2010). The strain was revived from frozen stock cultures (-80°C) on half-strength marine agar [ $\frac{1}{2}$ MA; 27.6 g Difco 212185 marine agar (Difco Laboratories, Detroit, MI, USA), 15 g sea salts (Instant Ocean, Vernon, Canada), 7.5 g agar, 1 l deionized water]. Plasmids pYedQ and pYhJH were obtained from Tim Tolker-Nielsen (University of Copenhagen) and electroporated into *R. mobilis* F1926, as described below. Half-strength yeast-tryptone-sea-salts broth ( $\frac{1}{2}$ YTSS) [2 g yeast extract (Bacto Laboratories, Sydney, Australia), 1.25 g tryptone (Bacto Laboratories, Sydney, Australia), 20 g sea salts (Sigma-Aldrich, St. Louis, MO, USA), 1 l deionized water] and agar (Gonzalez *et al.*, 1996) containing 50  $\mu$ g ml<sup>-1</sup> of tetracycline were used for selecting transconjugants after the electroporations and for routine culturing of the plasmid-carrying

**Table 3.** Bacterial strains and plasmids used in the present study.

Strain or plasmid	Genotype or relevant markers	Source or reference
<b>Strain</b>		
<i>Ruegeria mobilis</i> F1926	Wild type	Gram <i>et al.</i> 2010; L. Gram, P. D'Alvise, C. Porsby, J. Melchiorson, J. Heilmann, M. Jensen, et al., unpubl. data
<i>E. coli</i> TransforMax EC100D <i>pir</i> <sup>+</sup>	<i>pir</i> <sup>+</sup>	Epicentre
<b>Plasmids</b>		
pRK415	Conjugative broad host range vector, Tet <sup>R</sup>	Keen <i>et al.</i> 1988
pPDA11	<i>tdaCp::gfp</i> ligated into pRK415, Tet <sup>R</sup>	This study; D'Alvise <i>et al.</i> 2012
pYedQ	<i>E. coli</i> gene <i>yedQ</i> (diguanylyl cyclase) ligated into pRK404A, Tet <sup>R</sup>	Ausmees <i>et al.</i> 2001; Gjermansen <i>et al.</i> 2006
pYhjH	<i>E. coli</i> gene <i>yhjH</i> (c-di-GMP-specific phosphodiesterase) ligated into pBBR1MCS-3, Tet <sup>R</sup>	Gjermansen <i>et al.</i> 2006
pRK404A	Standard broad host range cloning vector, Tet <sup>R</sup> , control for pYedQ	Ditta <i>et al.</i> 1985
pBBR1MCS-3	Standard broad host range cloning vector, Tet <sup>R</sup> , control for pYhjH	Kovach <i>et al.</i> 1995

mutants. Cultures for microscopy and for chemical measurements of c-di-GMP were grown in full strength MB that contained 50 µg ml<sup>-1</sup> of tetracycline for the plasmid-carrying mutants. For inhibition testing and TDA analysis, all strains were cultured in MB without addition for 3 days. A 1.5 YTSS with and without tetracycline was used for biofilm and attachment assays. All cultures were grown as 20 ml batches in 250 ml glass bottles at 25°C, except as noted otherwise, and shaking velocity was 200 r.p.m.

#### Genome sequencing

Genomic DNA was obtained from strain F1926 by successive phenol-chloroform purification steps (Sambrook and Russel, 2001). Mate pair library preparation and Illumina Hi Seq 2000 (Illumina, San Diego, CA, USA) sequencing were carried out by the Beijing Genomic Institute (Shenzhen, China). Contigs were assembled using CLC Genomic Workbench (CLC Bio, Aarhus, Denmark). The genomic DNA sequence has been submitted to the National Center for Biotechnology Information (NCBI) database under accession number AQCH00000000.1.

#### Detection of genes with GGDEF and EAL domains

The genome draft was annotated by NCBI using the PGAAP, and genes encoding diguanylate cyclases and phosphodiesterases were identified (Table 1). The sequences of these genes were used to search the Pfam database (Punta *et al.*, 2012), and the e-values of the Pfam-identification of the GGDEF- and EAL-domain proteins are stated in Table 1.

#### Electroporations

The electroporation method was adapted from Miller and Belas (2006). Recipient cells were grown in 50 ml ½YTSS (*R. mobilis*) or LB medium (*E. coli*, 37°C) until OD600 was about 0.5, chilled on ice for 30 min, harvested by centrifugation at 2380 × g, washed twice in 10 ml autoclaved, ice-cold

MilliQ-water (Merck Millipore, Billerica, MA, USA) and resuspended in 0.5 ml ice-cold 10% glycerol. Aliquots of 70 µl were stored at -80°C until use. Electrocompetent cells were mixed with 180–230 ng plasmid DNA, incubated 30 min on ice, transferred to a 0.2 cm electroporation cuvette (165–2086 Biorad, Hercules, CA, USA) and electroporated at 2.5 kV cm<sup>-1</sup>, 200 Ω, 25 µF using a Biorad Gene Pulser (Biorad). The cells were immediately transferred to liquid growth medium without antibiotics, recovered for 2–4 h and plated on selective agar.

#### c-di-GMP extraction

Shaken and static cultures of *R. mobilis* F1926 wild type and the plasmid-carrying mutants were inoculated from OD-adjusted overnight precultures and grown for 24 h in triplicates. The cultures were cooled on ice, and static cultures were shaken briefly to break up the biofilms. One millilitre was sampled and vortexed vigorously to further break up aggregates before measuring OD600. Cultures were harvested (5000 × g), and the pellets were extracted with 10 ml 75% (v/v) boiling ethanol/water containing 10 µM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. The pellets were resuspended in 75% ethanol, and the suspensions were left in an 80°C water bath for 5 min. Subsequently, the suspensions were centrifuged (4250 × g), and the supernatants were evaporated to dryness under nitrogen. The samples were dissolved in 100 µl mobile phase A and filtered through 0.2 µm PTFE (polytetrafluoroethylene) hydrophilic filters before analysis.

#### Ion pair UHPLC-MS/MS analysis of c-di-GMP

The analysis was carried out on an Agilent 1290 (Agilent, Torrance, CA, USA) binary UHPLC system coupled with a 6460 triple quadrupole mass spectrometer (Agilent). The MS was operated in negative electrospray using the [M-H]<sup>-</sup> m/z 689.1 as parent ion, and m/z 149.9 and 537.9 as quantifier and qualifier ions respectively. Separation of 10 µL samples was performed by ion pair chromatography, as described



in detail in (Magdenoska *et al.*, 2013), using 10 mM tributylamine as ion pair reagent. The gradient used was 0–12 min 0–50% B, 12–12.5 min 50–100% B, 12.5–13 min 100% B, 13–13.1 min 100–0% B, 13.1–18 min 0% B. Three hundred and sixty milliliters of shaken cultures of the pYhjH-carrying mutant were split into eighteen 50 ml falcon tube and used for matrix-matched calibration. One mg/ml c-di-GMP in water was used to prepare the spiking solutions. After centrifugation and removal of the supernatants, 75% boiling EtOH was added to the tubes followed by spiking with, 100 µl of 0 ng/ml, 35 ng/ml, 100 ng/ml, 200 ng/ml, 600 ng/ml and 1000 ng/ml c-di-GMP standard in triplicates. The spiked cultures were extracted and prepared for analysis as described above. The amount of c-di-GMP detected in the blank was subtracted from the spiked calibrants, and the analysis was calibrated by linear regression ( $r^2 = 0.993$ ). To obtain a relative estimate of c-di-GMP concentrations per cellular biomass, c-di-GMP concentrations were divided by the measured OD600 of the original cultures.

### Biofilm and attachment assay

Biofilm formation in *R. mobilis* F1926 wild type, F1926 pYedQ and F1926 pYhjH was measured by a crystal violet method (O'Toole *et al.*, 2000). Briefly, shaken precultures were diluted with fresh medium to an OD600 of 0.1, pipetted into a 96-well microtiter plate and incubated for 24 h. Culture liquid was removed, and biofilms were washed and stained in 1% (w/v) crystal violet solution. After washing, the crystal violet was extracted from the stained biofilms with 96% ethanol and quantified by measuring absorption at 590 nm.

Attachment to an inert surface was measured in a modified crystal violet assay. Static cultures were grown in 96-well plates as described above. Shaken cultures were grown in glass bottles for 24 h; OD600 was adjusted to 1.0, and 200 µl were pipetted into the wells of a microtiter plate. A lid with 96 polystyrene pegs (Innovotech, Edmonton, Canada) was placed on the plate, and the cells were allowed to attach to the pegs for 1 min. Adherent biofilms on the pegs were washed twice by dipping into water, dried for 5 min and stained in crystal violet solution. After triple washing in water, the crystal violet was extracted from the stained biofilms on the pegs in each 200 µl of ethanol, and absorption was measured at 590 nm. Both assays were conducted in duplicates and reproduced independently.

### Measurement of antibacterial activity

Inhibition of *Vibrio anguillarum* 90-11-287 in a standard well-diffusion assay was measured as an approximation to TDA production as adapted from (Hjelm *et al.*, 2004). *V. anguillarum* 90-11-287 was grown in MB for 1 day at 25°C with aeration at 200 r.p.m. A 50 µl of the *V. anguillarum* preculture was added to 50 ml molten Instant Ocean agar [1.5 g of Instant Ocean sea salts, 0.1 g of casamino acids (Bacto Laboratories, Sydney, Australia), 0.2 g glucose, 0.5 of g agar] at 41.5°C and poured into a 14 cm Petri dish. Wells of 6 mm diameter were punched into the solidified agar and filled with 50 µl of *R. mobilis* F1926 culture supernatant. The assay was incubated for 1 day at 25°C, and diameters of inhibition zones were measured. The results are based on two independent replicates.

### TDA extraction and analysis

A 1 ml of each MB culture was mixed with 3 ml ethyl acetate containing 1% formic acid and extracted for 30 min on a shaking table at room temperature. A 2.5 ml of the organic phase was evaporated to dryness under nitrogen flow at 35°C and redissolved in 100 µl 85% acetonitrile/15% MilliQ water. Blank medium samples spiked with 0.13–100 µM pure TDA (BioVotica, Dransfeld, Germany) served as standard. UHPLC-TOFMS analysis was conducted on an Agilent 1290 UHPLC coupled to an Agilent 6550 qTOF (Agilent) equipped with a dual electrospray source. Separation was performed at 40°C on a 2.1 mm ID, 50 mm, 1.8 µm of Eclipse Plus C<sub>18</sub> (Agilent) column using a water-acetonitrile gradient solvent system, with both water and acetonitrile containing 20 mM of formic acid. Using a flow of 0.8 ml/min, the gradient was started at 15% acetonitrile and increased to 60% acetonitrile within 1.8 min, then to 100% in 0.2 min, keeping this for 0.8 min, returning to 15% acetonitrile in 0.2 min and equilibrating for the next sample in 1.5 min (total runtime is 4.5 min). TDA was determined in ESI<sup>+</sup> mode and quantified from its [M + H]<sup>+</sup> ion  $212.9674 \pm 0.005$  with the same retentions as the authentic standard (0.97 min). Quantification was done using 1/x weighted linear regression based on the peak area in the MASSHUNTER QUANT 5.0 software (Agilent). Duplicate cultures were used for TDA analysis, and the experiment was reproduced independently.

### Statistics

Cellular c-di-GMP concentrations and TDA concentrations were compared by *t*-tests. Differences in average crystal violet absorption values in the biofilm and attachment assays were examined by one-way ANOVA with Tukey's multiple comparison test using the software PRISM version 4.03 (GraphPad Software, La Jolla, CA, USA). All average values and standard deviations are based on biological replicates.

### Construction of a *tdaCp::gfp* reporter fusion

A transcriptional fusion between the promoter of *tdaC* and a promoterless *gfp* gene was constructed similarly to pHG1011 (Geng and Belas, 2010a). The promoter sequence of the *tdaC* gene was amplified using the primers ptdacF (5'-GTCCCAGAGACCAACGCAATGAGTAAAGGAGAAGAA-3') and ptdacR (5'-TTCTTCTCCTTTACTCATTGCGTTGGTCTCTGGGAC-3'). The *gfp* open reading frame in pAKN137 (Lambertsen *et al.*, 2004) was amplified using the primers gfpF (5'-GTCCCAGAGACCAACGCAATGAGTAAAGGAGAAGAA-3') and gfpR (5'-TGATAAGCTTTTATTTGTATAGTTTCATCCATGCCATGT-3'). Primer ptdacF created a PstI-restriction site, and gfpR created a HindIII-site. Primer ptdacR and gfpF created identical 36-bp-sequences in the adjacent ends of the two amplicons, each containing the end of the promoter and the start of the *gfp* open reading frame. This allowed seamless cloning of promoter and *gfp* gene by overlap-extension polymerase chain reaction. The product was cloned into the broad-host range vector pRK415 (Keen *et al.*, 1988) after both had been digested with PstI and HindIII (New England Biolabs, Ipswich, MA, USA) to yield plasmid pPDA11.

### Microscopy

Shaken and static cultures of *R. mobilis* F1926 wild type and the mutants carrying pYedQ and pYhjH were grown in duplicates for 24 h and were compared by phase contrast microscopy. Before specimen preparation, static cultures were agitated briefly to break the biofilms into smaller pieces for sampling. Images that were representative of the specimen were recorded. Screening the whole specimen, rosette formation, and the proportion of single cells and cells in biofilms were registered. The proportion of motile cells was estimated visually. Absence of motile cells or rosettes was stated if no motile cell or rosette was observed in either of the duplicate samples.

*Gfp*-fluorescence of *R. mobilis* F1926 pPDA11 was detected by microscopy using a long-pass fluorescence cube (Olympus, Tokyo, Japan; WIB ex. 460–490, em. > 515). Shaken and static cultures were grown for 3 days. Again, static cultures were briefly shaken before specimen preparation. Representative fluorescence micrographs were recorded with 1.5 s exposure, and a phase contrast image of the same area was recorded right thereafter. To record a time series showing the onset of *gfp* expression in response to attachment, a 3 day old shaken culture was diluted 1:2 with fresh medium and grown for 4 h at the same conditions. A specimen was prepared, and a time series of fluorescence micrographs was recorded as described above. For better display, contrast of the phase, contrast pictures and brightness of the fluorescence micrographs were enhanced using Photoshop (Adobe, San Jose, CA, USA). The same adjustments were made on all images of the same type.

### Motility agar

Motility agar was prepared based on half-strength and 1/10-strength YTSS (0.4 g Bacto Yeast extract, 0.25 g Bacto Tryptone, 20 g Sigma sea salts, 1 l deionized water) with different agar percentages (0.5, 0.45, 0.4, 0.35, 0.3 and 0.2%) and 5 ml/l 1% tetrazolium red solution, and Stab agar tubes and Petri dishes were prepared. The Petri dishes were inoculated with 5 µl of OD-adjusted (OD<sub>600</sub> = 0.5) 1.5 YTSS precultures, and the stab agar cultures were inoculated with a needle dipped into the same OD-adjusted precultures. The diameters of growth were compared after 1, 2 and 5 days.

### Acknowledgements

We thank Tim Tolker-Nielsen (University of Copenhagen) for providing plasmids pYedQ and pYhjH, as well as the vector controls, and we thank Jens Bo Andersen (University of Copenhagen) for technical advice with the construction of plasmid pPDA11. We are grateful to Agilent technologies for the Thought Leader Donation of the UHPLC-qTOF system.

This work was funded by the Danish Research Council for Technology and Production Sciences (project 09-066524).

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### Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Phylogenetic tree of *Ruegeria mobilis* constructed from 16S rRNA gene sequences. *R. mobilis* F1926 is compared with the *R. mobilis* type strain NBRC 101030 (accession number NR\_041454), other *R. mobilis* isolates (accession numbers HQ338144.1, HQ338146.1, HQ338142.1, HQ338140.1, HQ338148.1, HQ338141.1, HQ338145.1, HQ338143.1) and other species from the *Ruegeria/Phaeobacter* subcluster (Newton *et al.*, 2010) of the *Roseobacter* clade (accession numbers NR\_074151.1, NR\_042675.1, NR\_043449.1, GU176618.1, HQ\_908721.1, NR\_029273.1, NR\_027609.1, NR\_042761.1, AJ536669.1,

NR\_074150.1, FJ872535.1). *Rhodobacter capsulatus* ATCC11166 and *Rhodobacter sphaeroides* ATCC BAA-808 (accession numbers DQ342320.1 and NR\_074174.1) served as outgroup. The 16S sequences were aligned using ClustalW (Thompson *et al.*, 1994), and the neighbour-joining tree was constructed using MEGA version 5 (Tamura *et al.*, 2011). Numbers at the nodes are bootstrap values from 500 replications.

**Fig. S2.** Cyclic di-GMP detection in extracts of *Ruegeria mobilis* F1926. Example chromatograms of F1926 pYhjH shaken culture spiked with c-di-GMP (top) and F1926 wild type static culture (bottom). The MRM transition monitored is m/z 689.1→149.9. The counts on the y-axis are relative to the highest peak in the respective sample; thus, the figure does not allow for quantitative comparison.

**Table S1.** Guanylate cyclase and phosphodiesterase genes in TDA-producing and non-TDA-producing *Roseobacter* clade species.